Basal Cell Glycoprotein in Pig Epidermis Closely Resembles the β_1 Subunit of the Integrin Family of Cell Adhesion Molecules

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A 135-kD conA-binding glycoprotein isolated from pig epidermis was previously localized to the surface of basal cells in stratified epithelia using affinity-purified antibodies. Preembedding immunoperoxidase electron microscopy has now shown that this glycoprotein is concentrated on the lateral surfaces of basal cells but is not detectable on those surfaces adjacent to the basement membrane indicating a role in cell-cell rather than cell-substrate interactions. The basal cell glycoprotein was shown to resemble the β_1 subunit of the integrin family following the generation of a specific monoclonal antibody (M5.25). The epidermal glycoprotein recognized by M5.25 and by antibodies against the β_1 fibronectin receptor from human placenta co-migrated on SDS gels under both reducing and non-reducing conditions. Its response to disulphide reducing agents was characteristic of β_1 integrin subunits. In addition, the basal cell glycoprotein was shown to bind to the 120-kD cell-binding fragment of fibronectin in a RGD-dependent manner. It was readily detected by immunoblotting whole cell lysates of cultured pig keratinocytes suggesting increased expression in cultured cells compared to fresh epithelial tissue. The results suggest that β_1 integrin subunits may be involved in cell-cell interactions between basal keratinocytes in pig epidermis and that these receptors are lost from the cell surface during terminal differentiation. Thus modulation of β_1 integrin subunit expression may play an important role in regulating differentiation in pig epidermis. *J Invest Dermatol 97:501–505, 1991*

In order to study the role of cell-adhesion molecules in epidermal differentiation we have been isolating and characterizing some of the lectin-binding glycoproteins in pig and human epidermis [1–3]. Polyclonal and monoclonal antibodies have been generated against individual components and have been used to localize particular glycoproteins within the tissue.

In 1986 we described a 135-kD glycoprotein, isolated from particulate fractions of pig epidermis, that was restricted to the basal layer of the epidermis and other stratified epithelia [1]. This localization suggested a possible role either in the proliferative activity of the epithelium or in its attachment to the underlying connective tissue. Metabolic labeling with [^{3}H]glucosamine showed that this component was one of the major glycoproteins synthesized in pig epidermal explants. Immune-precipitation experiments showed co-precipitation of another glycoprotein with an Mr of 165-kD that could have been a precursor of the 135-kD form [1]. Alternatively the 165-kD and 135-kD components might have represented distinct glycoprotein subunits in a dimeric complex present at the surface of basal epidermal cells.

Following the recent characterization of the integrin family of cell-adhesion molecules as membrane glycoprotein dimers containing α subunits of 130 to 210 kD and β subunits of 95 to 130 kD [4–6], we have investigated the relationship of the 135-kD glycoprotein from pig epidermis to the integrin receptors. In this report we have shown that the basal-cell glycoprotein in pig epidermis closely resembles the β_1 subunit of the integrin family of cell adhesion molecules.

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Abbreviations:
conA: concanavalin A
SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis
FITC: fluorescein isothiocyanate
HRP: horseradish peroxidase

MATERIALS AND METHODS

Isolation of Particulate Glycoproteins Glycoproteins were isolated from the particulate fraction of $CaCl_2$-separated pig epidermis by lectin affinity chromatography on ConA as described previously [1].

Antibodies Affinity-purified antibodies against the 135-kD glycoprotein in particulate fractions from pig epidermis were described previously [1]. Rabbit antiserum against the human β_1 fibronectin receptor [7] was obtained from Calbiochem Corporation. The monoclonal antibody M5.25 was generated from splenocytes of mice immunized with conA-binding glycoproteins from the particulate fraction of $CaCl_2$-separated pig epidermis. Splenocytes were fused with Sp2 Ago/14 myeloma cells using 50% polyethylene glycol and were plated out [8] in azaserine-hypoxanthine selection medium (Sigma). Wells were screened by an ELISA assay using microtitre plates coated with particulate glycoproteins from pig epidermis [9]. Wells containing positive hybridomas were expanded and tested for reaction with the 135-kD glycoprotein by immunoblotting. Positive hybridiomas were cloned by limiting dilution in the presence of splenocyte feeder cells, rescreened, expanded, and cloned again and hybridoma supernatant was removed for further analysis. M5.25 was identified as an IgG1 using the Amersham isotyping kit.

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Indirect Immunofluorescence Five micrometer sections of pig and human skin that had been snap-frozen in isopentane/liquid N_2 [1] were air-dried and tested with undiluted M5.25 hybridoma supernatant and FITC-conjugated rabbit anti-mouse IgG (1:20, Dakopatts). Cryostat sections were examined directly after cutting or after storage at −70°C for up to 3 months.

Fibronectin Affinity Purification [10] The affinity matrix consisted of 2 mg of the 120-kD chymotryptic fragment of human fibronectin (Calbiochem Corporation) coupled to 2 ml of CNBr-activated Sepharose 4B (Pharmacia). The particulate fraction from 100 g of dermatomed pig ear skin slices (not $CaCl_2$-separated epidermis) was extracted with 20 ml of 0.1 M octylglucoside (Calbiochem Corporation) in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and the detergent extract was incubated with the fibronectin affinity matrix overnight at 4°C. The mixture was transferred to a small plastic column and the matrix was washed extensively with 0.05 M octyl glucoside in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. This column was eluted with 1 mg/ml of the hexapeptide GRGDSP (Novabiochem) in washing buffer. Portions of the total extract and the eluate were precipitated with 10 vol of ethanol at −20°C and resuspended in H_2O before determination of protein concentration.

SDS-PAGE and Immunoblotting SDS gel electrophoresis was performed on 7 or 8% polyacrylamide gels [11]. Reduced and non-reduced samples (with or without 5% 2-mercaptoethanol) were run simultaneously, but on separate gels, together with reduced or non-reduced high Mr standards (BioRad). Proteins were transferred to nitrocellulose [12] and either stained with amido black or probed with M5.25 hybridoma supernatant (neat to 1:10 dilution) followed by rabbit anti-mouse IgG (10 μg/ml) and then HRP conjugates of protein A or goat anti-rabbit IgG (1:200 dilution, BioRad). Alternatively nitrocellulose strips were probed with the β_1 integrin fibronectin receptor antiserum (1:100 to 1:500 dilution) followed by HRP goat anti-rabbit IgG (1:200 dilution, BioRad). Bound antibodies were detected using 4-chloro-1-naphthol (BioRad) as substrate.

Immunoperoxidase Electron Microscopy Slices of unfixed pig skin taken with a razor blade were incubated with affinity-purified antibodies against the 135-kD glycoprotein for 1 h at room temperature and were washed 3 times with 1% bovine serum albumin in PBS. The tissue was then incubated with HRP conjugated swine anti-rabbit IgG (1:20) for 1 h at room temperature and washed 3 times with PBS. After fixation in 2% glutaraldehyde in 0.1 M phosphate (overnight at 4°C) the tissue was washed and incubated with 0.03% 3,3 diaminobenzidine in 50 mM Tris HCl for 30 min at room temperature and further washed with PBS. Samples were fixed with 1% OsO_4, washed, dehydrated in graded ethanols and acetone, and infiltrated and embedded in Spurr resin. Sections were cut from the surface of the embedded skin slice to ensure that any reaction product was included.

Keratinocyte Cultures Keratinocytes obtained from trypsinized pig skin were cultured on 3T3 feeder cells as described previously for human keratinocytes [13]. Confluent cell layers were rinsed with PBS, scraped from the surface of the flask after the addition of SDS sample buffer and solubilized by heating at 100°C for 5 min. Samples were clarified by centrifugation before analysis by SDS-PAGE.

RESULTS

Affinity-Purified Antibodies Previous immunofluorescent studies with affinity-purified antibodies showed that the 135-kD conA-binding glycoprotein in pig epidermis was restricted to the surface of cells in the basal layer of the tissue [1]. More precise localization at the electron microscope level was hindered by the fixation-sensitivity of the glycoprotein. However, this glycoprotein has now been shown to be concentrated in areas of cell contact between keratinocytes using a pre-embedding immuno-peroxidase technique. The affinity-purified antibodies produced strong staining along the lateral plasma membranes of adjacent basal cells but failed to stain the basal plasma membrane apposing the basement membrane (Fig 1). This differential distribution suggested a possible role in keratinocyte-keratinocyte interactions rather than keratinocyte-substratum interaction in pig epidermis.

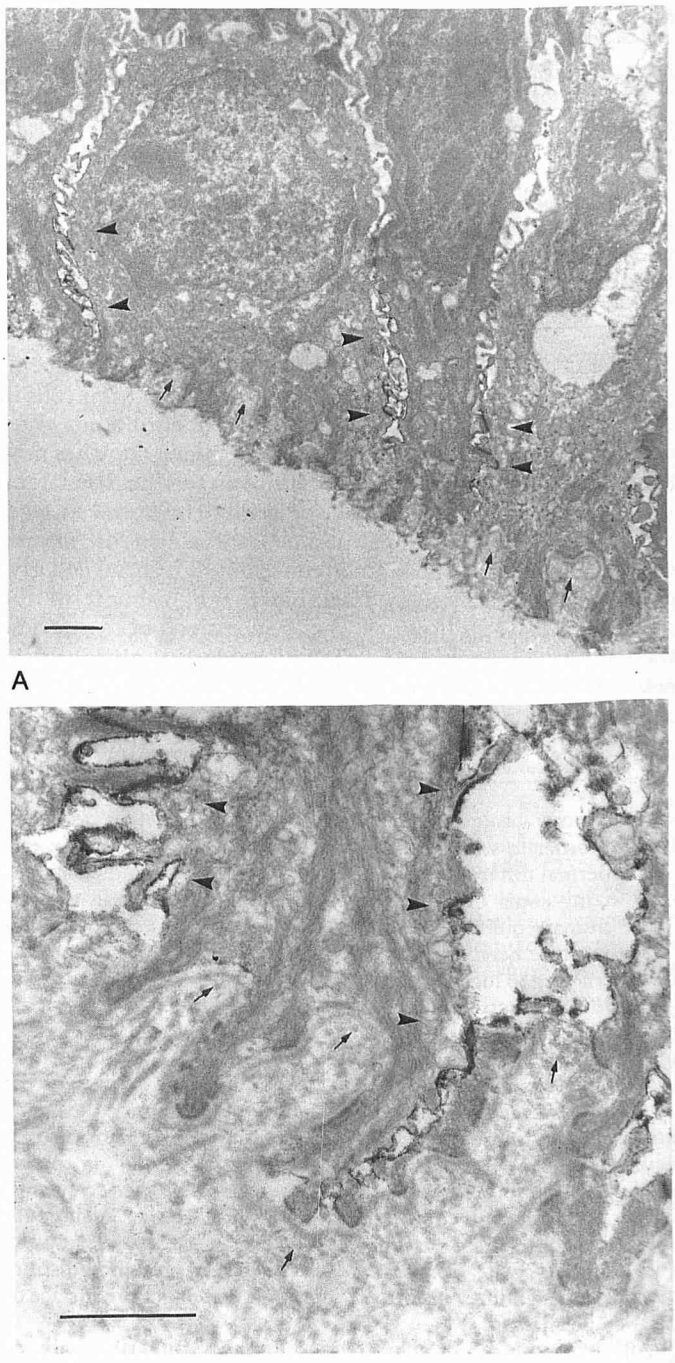

Figure 1. Immunoperoxidase staining of pig skin with affinity-purified antibodies against the 135-kD glycoprotein from pig epidermis. Low- *(A)* and higher-power *(B)* photomicrographs show deposition of reaction product along lateral surfaces of basal keratinocytes *(arrowheads)*. Note the absence of staining in membranes adjacent to the basement membrane *(arrows)*. *Bar*, 1 μm.

Monoclonal Antibody (M5.25) The yield of the 135-kD conA-binding glycoprotein from pig epidermis was very low (ap-

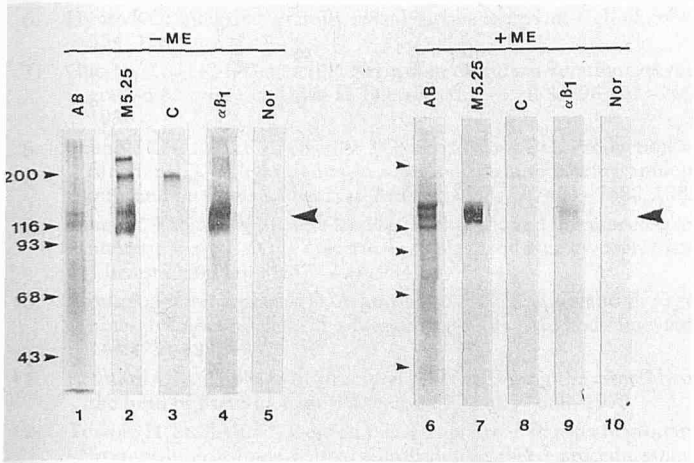

Figure 2. Immunoblotting of conA-binding glycoproteins from the particulate fraction of $CaCl_2$ separated epidermis run under non-reducing *(tracks 1–5)* and reducing conditions *(tracks 6–10)*. About 70 μg of protein was run in each track. After transfer, nitrocellulose strips were stained with amido black (AB, *tracks 1 and 6*) or probed with M5.25 (diluted 1:10 in *track 2*, undiluted in *track 7*), an unrelated IgG monoclonal (C, *tracks 3 and 8*), anti-human β_1 integrin serum ($\alpha\beta_1$, diluted 1:200, *tracks 4 and 9*) and normal rabbit serum (Nor, diluted 1:200, *tracks 5 and 10*). *Small arrowheads,* position of Mr standards (in kD); *large arrowhead,* position of the basal cell glycoprotein.

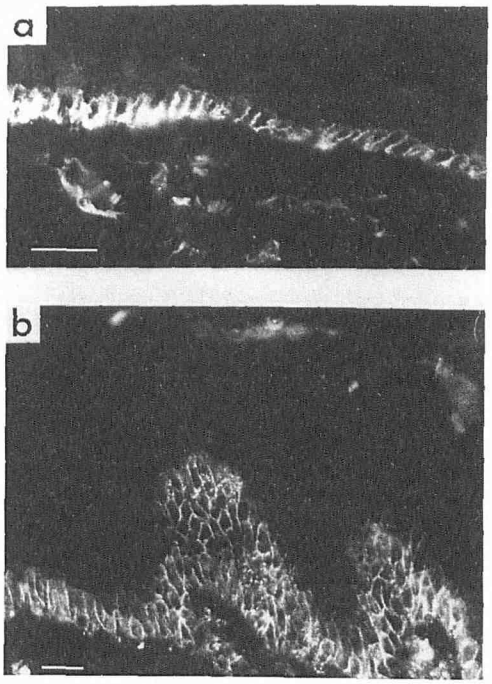

Figure 3. Indirect immunofluorescence of skin sections (that had been stored for several weeks at −70°C) with M5.25. Cell-surface staining is generally restricted to basal epidermal cells *(a)* but cell-surface staining expands into the lower spinous layers over rete ridge areas *(b)*. *Bar,* 20 μm.

proximately 5 μg/g tissue) providing limited material for the affinity-purification of specific antibodies. Therefore a monoclonal antibody (M5.25) was generated that recognized the 135-kD glycoprotein in SDS gels run under standard reducing conditions (Fig 2, *track* 7). Under non-reducing conditions (Fig 2, *track 2*) this glycoprotein migrated significantly faster as a broad band with an apparent Mr of 125 kD. This electrophoretic behavior is consistent with a compact structure stabilized by intrachain disulphide bonds as found with the β_1 integrin subunits. M5.25 reacted much more strongly with the non-reduced form of the glycoprotein suggesting that its epitope was also stabilized by disulphide bonds. Under non-reducing conditions M5.25 also recognized a band at about 250 kD, which would be the size expected of a disulphide cross-linked dimer (Fig 2, *track 2*). The reaction with a band at about 190 kD represented non-specific staining, because it was also observed with unrelated antibodies (Fig 2, *track 3*).

In immunofluorescence tests M5.25 never stained frozen sections obtained from fresh pig skin. However, staining was occasionally observed in sections that had been stored frozen at −70°C for several weeks (Fig 3) suggesting exposure of the M5.25 epitope in situ by oxidation. The immunofluorescent staining was identical to that seen with the affinity-purified antibodies [1]. It was restricted to the basal layer of the epidermis except over rete ridges where cell-surface staining extended into the lower spinous cell layers. Again those cell surfaces adjacent to the basement membrane were not stained.

Staining patterns similar to those seen with the affinity-purified antibodies and M5.25 have recently been reported for β_1 integrin subunits in human epidermis [7,14–16]. In other tissues, β_1 integrin subunits have a similar size to the basal cell glycoprotein from pig epidermis [6] and they also display similar electrophoretic responses to disulphide reducing agents [17]. The reaction of the basal cell glycoprotein with authentic antibodies against the β_1 fibronectin receptor was therefore examined. In non-reduced samples these antibodies recognized the same broad 125-kD band as M5.25 (Fig 2, *track 4*). After reduction, these human β_1 integrin subunit antibodies recognized the 135-kD glycoprotein band although the reaction was much weaker than with the non-reduced form (Fig 2, *track 9*).

To confirm that the basal cell glycoprotein in pig epidermis was functionally related to the β_1 integrin subunit octylglucoside extracts of a particulate fraction were incubated with an affinity matrix containing the cell-binding fragment of fibronectin [17]. After washing, the affinity column was eluted with a peptide containing the RGD adhesion sequence. This treatment eluted the basal cell glycoprotein recognized by both M5.25 and the human β_1 integrin subunit antibodies (Fig 4, *tracks 5 and 6*) confirming that it had bound to fibronectin via RGD recognition sequences as expected of a β_1 integrin subunit.

The β_1 subunit-like glycoprotein appeared to be expressed more abundantly in cultured pig keratinocytes than in pig epidermal tissue. It was readily detected by immunoblotting whole cell lysates solubilized in SDS sample buffer (Fig 5, *tracks 2 and 4*) without prior isolation of the glycoprotein by lectin or fibronectin affinity chromatography. This abundance is consistent with the up-regulation of fibronectin receptors in culture as suggested by recent studies of cultured human keratinocytes [18].

DISCUSSION

The 135-kD glycoprotein from pig epidermis was originally characterized as a basal cell glycoprotein using affinity-purified antibodies [1]. Further studies of this component were frustrated by the fixation sensitivity of the glycoprotein and the low yields obtained from tissue that limited the amounts of purified antibody that could be prepared. Although the generation of a specific monoclonal antibody provided a useful advance its failure to stain fresh tissue cast some doubt on its general usefulness as a reagent. However the observations that the M5.25 epitope was exposed during prolonged storage of tissue sections (presumably by oxidation) and the increased staining and increased migration of the non-reduced glycoprotein all suggested that the glycoprotein had an unusual structure possibly influenced by intrachain disulphide bonding as reported for β_1 integrins [17].

The 135-kD basal cell glycoprotein was shown to closely resemble the β_1 subunit of the integrin family of cell adhesion molecules by comigration with the glycoprotein recognized by antibodies against the human receptor under both reducing and non-reducing

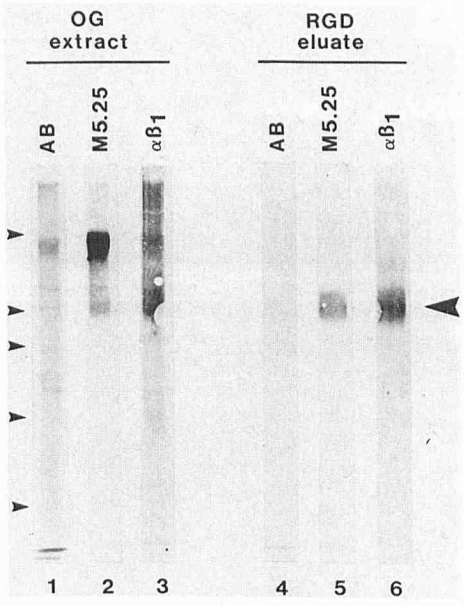

Figure 4. Immunoblotting of octylglucoside (OG) extract (about 50 μg of protein track) from the particulate fraction of pig skin and the material eluted from a fibronectin affinity matrix by GRGDSP (about 5 μg of protein/track). The gel was run under non-reducing conditions. After transfer, nitrocellulose strips were stained with amido black (AB, *tracks 1 and 4*) or probed with M5.25 (undiluted *tracks 2 and 5*) or anti-human β_1 integrin serum ($\alpha\beta_1$, diluted 1 : 100, *tracks 3 and 6*). *Arrowheads* as in Fig 2.

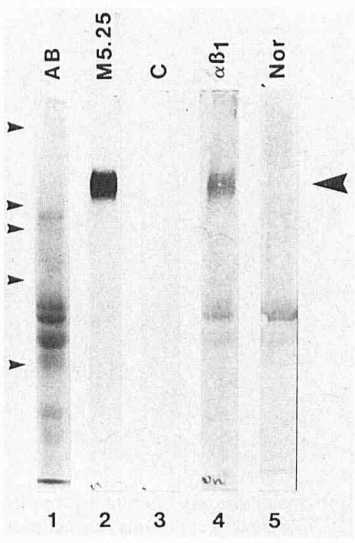

Figure 5. Immunoblotting of whole cell lysate from cultured pig keratinocytes run under non-reducing conditions (about 75 μg protein/track). After transfer nitrocellulose strips were stained with amido black (AB, *track 1*) or probed with M5.25 (undiluted, *track 2*), an unrelated, control, IgG monoclonal (C, *track 3*), anti-human β_1 integrin serum ($\alpha\beta_1$, diluted 1 : 100, *track 4*), and normal rabbit serum (nor, diluted 1 : 100, *track 5*). *Arrowheads* as in Fig 2.

conditions. This was confirmed by demonstration of its RGD-dependent binding to fibronectin. The pig glycoprotein appears to be somewhat larger than the receptors isolated from other sources, which each have a reported Mr of 120 to 130 kD [5]. This might reflect differences in post-translational modification but could equally reflect minor differences in electrophoresis techniques. However, other results suggest possible differences in glycosylation. The β_1 subunit-like glycoprotein from pig epidermis was isolated by lectin chromatography on conA, whereas the receptor from human placenta and human osteosarcoma cells was bound by wheat germ agglutinin but not by conA [17]. It remains to be seen whether these glycosylation differences reflect species specificity and/or tissue specificity. A recent study found that the mature β_1 integrin subunit in human keratinocytes is about 5 kD larger than that from dermal fibroblasts [15].

Our tissue localization studies suggest that the keratinocyte β_1 integrin subunit is not concentrated on those cell surfaces involved in attachment of the epithelium to the basement membrane. It is more prominent on those regions of the plasma membrane involved in cell-cell interactions between basal keratinocytes. This suggests a role in cell-cell rather than cell-substratum adhesion in pig epidermis and is in agreement with recent findings in human keratinocytes [15]. A monoclonal antibody against the human β_1 integrin subunit and its Fab fragments were found to dissociate human keratinocyte colonies growing as a monolayer in low Ca^{++} conditions. However, these reagents had no apparent effect on the cells' adhesion to substrate. This appeared to be a direct effect on keratinocyte cell-cell interactions. It was independent of any effects on cell-substrate interactions, as similar results were obtained in low Ca^{++} cells growing on vitronectin where integrins other than β_1 are involved in substrate attachment. It would clearly be of interest to determine whether the monoclonal antibody described here has similar dissociative effects on pig keratinocytes grown in low Ca^{++} conditions.

The present results show that terminal differentiation in pig epidermis is associated with the loss of a β_1 integrin subunit-like glycoprotein from the cell surface. Recent studies of human keratinocytes suggest that loss of these receptors may be preceded by a reduction in their ability to bind fibronectin [19]. Further, fibronectin has been shown to inhibit the suspension-induced terminal differentiation of human keratinocytes (assessed by changes in involucrin expression) via integrin receptors [20]. Although these studies were performed on cultured keratinocytes where integrin receptors may be up-regulated [18], they point to an important role for β_1 fibronectin receptors in the regulation of epidermal differentiation in vivo. Activation of β_1 integrins may be particularly relevant to the re-epithelialization process that occurs during wound healing. Changes in the level and distribution of β_1 receptors have been described during the migration of keratinocytes out of human skin explants [7]. Moreover, our earlier report showed that the β_1 integrin subunit was one of the major glycoproteins synthesized in the migrating epidermis of pig skin explants [1].

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